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Research Article

Possible effects of probiotic strains on suppression of *Vibrio* and enhancement of growth in rotifer, *Brachionus plicatilis*

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Abstract

Because of the importance of rotifers as an adequate live food in larviculture, this study was conducted to evaluate probiotic potency of four microorganisms, *Candida parapsilosis*, *Pseudoalteromonas flavipulchra*, *Lactobacillus sakei* and *Bacillus natto* on suppression of *Vibrio* species and increasing population growth, enzymes activity and nutrients retention in the rotifer, *Brachionus plicatilis*. Four experimental and a control treatment, each with four replicates, with the concentration of 10^8 CFU/mL^{-1} for each strain were applied. The results demonstrated that *L. sakei* and *B. natto* successfully suppressed *Vibrio* in experimental treatments and increased population growth and nutrients retention. The longevity of *B. natto* was recorded as the best in long-term inoculation than other treatments and the control in a way that after 8 days of rotifers starvation, the count of *B. natto* increased. The findings showed that the two bacteria, *B. natto* and *L. sakei*, appear to be a promising probiotic for rotifers in suppressing *Vibrio* and also in increasing population growth, nutrients retention, enzymes activity and long-term inoculation in rotifers.

Keywords: Rotifer, Suppression, *Vibrio*, Enzyme, *Brachionus*, Probiotic.

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Introduction

Spread of *Vibrio* in mariculture farms showed the highest records in comparison with other areas of aquaculture like ornamental or freshwater fish farming. Massive contamination of aquatic animals with *Vibrio spp.* could be because of the high dependency of mariculture on live prey. Apart from all benefits of live food like proper size, high nutrients, availability, and other essential properties, it is a major carrier of bacteria, whether pathogenic or beneficial. The effects of live food enrichment, with different species and strains of microbes, on their growth rate, survival rate, and enzyme activity is studied (Jafaryan *et al.*, 2011; Sahandi *et al.*, 2013). Probiotics possess several essential properties, including efficient adherence to the intestinal epithelial cells to reduce or prevent the colonization of pathogens (Bomba *et al.*, 2002; Jafaryan *et al.*, 2011), aggressive growth (Gomez-Gil *et al.*, 1998; Mahious *et al.*, 2006) and production of metabolites to inhibit or kill pathogens (Helland *et al.*, 1996, De-Schrijver and Ollevier, 2000; Yu *et al.*, 2012). The efforts are carried out to promote strategies for microbial control (Jafaryan *et al.*, 2014). Probiotic supplements have particular relevance for development of rearing technologies for larviculture purposes. Administration of probiotics aims to induce health benefits for the host organism with improvement of enzyme secretion (Waché *et al.*, 2006; Sahandi *et al.*, 2012). The use of probiotics for aquatic animals is gaining much attention in the scientific community considering their potential benefits, such as controlling disease, enhancing immune response, providing nutritional and enzymatic contributions to the digestion of the host, and improving water quality. Probiotics can be supplied directly through the feed to introduce live cells of probiotics to the host animal. Selection of probiotic bacteria is a critical aspect which must be considered for the application of them.

Despite acceptance of probiotic bacteria's benefits in aquaculture, many questions remain unanswered, such as the effect of probiotics on pathogenic bacteria and possibility of pathogens suppression. Intensive rotifer culture using continuous or periodic addition of selected bacteria is found to enhance growth and modify healthy microbiota of rotifers (Planas *et al.*, 2004; Sahandi and Jafaryan, 2011). Live preys (rotifers and *Artemia*), with their non-selective feeding system, are essential carriers of contaminated substances into the larval digestive tract (Muroga *et al.*, 1987; Sahandi *et al.*, 2012; Ringo and Birkbeck, 1999), which significantly affect the microbiota of the larvae and, in many cases, is responsible for significant mortality rates (Muroga *et al.*, 1987). Rotifers (*Brachionus spp*.) are used as live food in feeding marine fish larvae for over 30 years (Yúfera, 2001). Today, more than 78 marine species require adequate and reliable production of high-quality, nutritious rotifers. The success of rotifers mass culture is determined not only by reproduction rate and density but also by their nutritional composition and their associated microbiota (Dhert *et al.*, 2001; Yúfera,

2001; Sahandi *et al.*, 2019). *Brachionus plicatilis* is the most cultured rotifer species in mariculture hatcheries.

Similarly, the microalgae *Nannochloropsis oculata* is a commonly-used species for rotifer cultivation because of its high material composition and small size. Considering the mentioned background and future needs, this study was designed and carried out to evaluate the effects of different bacteria and yeast strains on suppression of *Vibrio* species in rotifers. However, there was an interest in estimating the population growth, body composition, and digestive enzymes activity as well.

Material and methods

Microbial strains and culture condition

Four microorganisms, including *Candida parapsilosis*, *Pseudoalteromonas flavipulchra, Lactobacillus sakei, Bacillus natto* were selected according to their suppressive ability towards *Vibrio* species (Sahandi *et al.*, 2019). Suitable culture media were obtained based on the type of microorganisms including YPD for *C. parapsilosis*; TSB for *B. natto*; MRS for *L. sakei* and E2216 for *P. flavipulchra*. For culture of each strain, six test tubes were sterilized in an autoclave (Seisakusyo, Kagoshima-Japan) at 115˚C temperature for 30minutes and filled with proper culture medium. Then the test tubes with culture medium were inoculated with each strain under sterile condition. The bacteria and yeast were incubated in a rotary incubator (HDL, Model HZQ-F160, Shanghai-China) at 30°C overnight.

Microalgae culture

Microalgae (*Nannochloropsis oculata*) specimens were obtained from the College of Marine Life Science, (OUC, Qingdao, China), and were cultured using f/2 culture medium (Guillard, 1975). Culture was prepared with addition of 20 mL of algae into 70 mL of autoclaved seawater, which was maintained in a series of conical flasks after Balachandar and Rajaram (2018). The prepared cultures were kept at 28˚C with 2000lux illumination and cultured until reaching the density of 10^4cell/mL. Then the flask was replaced with a new culture for permanent access to fresh microalgae (Sahandi and Jafaryan, 2011).

Rotifer culture

Stock culture the rotifer, *Brachionus plicatilis*, was obtained from the Institute of Oceanography, Qingdao Agricultural University, (Qingdao, China). The first stock was kept in 1L flasks containing 800mL of seawater with 31‰ salinity, 28˚C temperature, 12:12 h light/dark cycle and permanent aeration. The primary inoculum of rotifer culture was 50rotifers/mL, with an initial 15–20 percent of ovigerous females. Rotifers were fed daily on microalgae (*Nannochloropsis oculata*) at a density of 10^4cell/mL (Sahandi *et al.*, 2012).

Experimental design

Determination of loading of Vibrio in rotifers

This experiment was conducted with four experimental treatments and one control (no addition of microbe) group, each with four replicates. The concentration of 10^8

CFU/mL of each strain was obtained and added into the rearing water at time zero and then after 5, 10, 15 and 20 hours of enrichment. Count of *Vibrio* species was estimated after Mahious *et al.* (2006). Proper samples were collected from each treatment, and biomass of collected rotifers was weighed, and then disinfected by benzalkonium chloride for 30 seconds to remove all the adhered microorganisms from the body surface. The disinfected samples were homogenized and serially diluted until 10^{-7} with autoclaved saline solution (0.9%, w/v) and from each dilution 100µL was spread on TCBS agar plates. The plates were incubated at 28˚C overnight, and the count of them was measured based on the colony-forming unit (CFU).

Growth indices

Population growth of each treatment was monitored every day to estimate the population changes. At the end of the trail, survival rate, rate of population increase expressed in days and rotifers generation time (g.t.) in days were calculated using the following equations:

Survival rate:

$s = [(N_0-N_t)/N_0]*100$

(Felix and Sudharsan, 2004)

Rate of population increase:

$\boldsymbol{r} = (\ln N_t - N_0)/t$ (Krebs, 1985)

Where $\boldsymbol{r}$ stands for rate of population increase; $\boldsymbol{N_t}$ stands for final count of rotifer; $\boldsymbol{N_0}$ stands for initial count of rotifer and $\boldsymbol{t}$ stands for time (day).

Generation time in days:

$\boldsymbol{g.t.} = t. \ln (N_t / N_0)$ (Krebs, 1985)

Where $\boldsymbol{g.t.}$ stands for generation time; $\boldsymbol{N_t}$ stands for final count of rotifer; $\boldsymbol{N_0}$ stands for initial count of rotifer and $\boldsymbol{t}$ stands for time (day).

Determination of digestive enzymes activity

In order to study the effect of applied strains in rotifer digestive enzyme activity, proper sampling was conducted at the end of the trail (seven days). The collected samples from each treatment (four replicates) were pooled, and then weighted and used for estimation of enzyme activity. Each weighted sample was homogenized at 4°C in sterile saline solution (8.5g/L) with a hand-held glass homogenizer which was placed in ice-bath. The suspension was then centrifuged with a refrigerated centrifuge (Eppendorf AG 22331, Hamburg, Germany) at 5000g for 20min at 4°C. The obtained supernatant was removed to assay the digestive enzymes activity. Protease activity was determined after Anson (1938), amylase activity was measured after Bernfeld (1955) and lipase activity was determined after Ota and Yamada (1966).

Determination of chemical composition of rotifers

Chemical composition determination of rotifers was performed after the feeding trial. The samples from each treatment were pooled. Moisture content was determined by weight loss after being freeze-dried. Crude protein content was calculated from the total nitrogen content, as described in AOAC (2005). Crude lipid content was measured in dry samples by the submersion method using boiling

hexane (Randall 1974), adapted for the Soxtec automatic system. Crude ash content was determined by further heating and weighing dry samples in thermogravi

metric analyzer at 600°C to constant weight for 8 hours. Carbohydrate content was calculated by the differences between dry weight and sum of crude protein, lipid, and ash contents as described by Kibria *et al.* (1999).

Determination of microbial longevity in rotifers

Sufficient count of rotifers from each replicate was randomly collected and transferred to the sterilized beakers which were filled with 100mL of autoclaved seawater (121˚C, 15psi, and 30min). The rotifers were starved for 8 days. Microbial composition of different treatments was estimated after two, four, and eight days of starvation of rotifers. Rotifers were filtered with 70µm mesh and washed with autoclaved distilled water. Then weighted and homogenized using a glass homogenizer (LeaMaster *et al.*, 1990) and plated after Mahious *et al.* (2006).

Fluorescent labeling of microbial strains

For finding out the form of probiotics accumulation in rotifers, the selected strains, including *P. flavipulchra, L. sakei*, *B. natto,* and *C. parapsilosis,* were labeled by DNA fluorescent stains (Bisbenzimide Hoechst 33342; Sigma-Aldrich, Darmstadt-Germany). The labeling procedure was carried out after Sahandi *et al.* (2019). Then the total count of 50 individuals of rotifers was transferred into the culturing cells with a capacity of 15mL each with three replicates. The labeled bacteria and yeast strains were added into each cell with a concentration of 10^8CFU/mL, and the rotifers were enriched for 3 hours. During the three hours of this test, different samples were randomly obtained. The samples were then prepared, one after another, and placed on the stage of fluorescence microscope with the upright position, and several micrographs were taken by using fluorescent light (Echo, San Diego, USA; x40).

Statistical analyses

Data was analyzed as a completely randomized design using the SPSS software version 21. One way ANOVA was performed. Tukey's multiple range tests were used to identify significant differences among treatments (p<0.05).

Result

The effect of selected beneficial strains on suppression of *Vibrio* species in rotifers was analyzed by plating, and the results are presented in Table 1. The results confirmed that the enriching time is a valid parameter on decrease or increase of *Vibrio* species, and each strain has its potential for long-term inoculation. The results showed that using selected strains reduced the concentration of *Vibrio* (p<0.05).

Table 1: Mean value (±SE) of *Vibrio* count in rotifer, *Brachionus plicatilis* which was enriched with different bacteria and yeasts over different times after hatching without benzalkonium chloride, Different letters in the same column indicate significant differences (p<0.05), the lowest score express the lowest loading rate of *Vibrio* on rotifer, *Brachionus plicatilis.*

	10^7 CFU/g rotifer			
	5 hours	**10 hours**	**15 hours**	**20 hours**
Control	12.13±0.38^{d}	12.64±0.32^{d}	12.2±0.55^{d}	14.09±0.32^{d}
C. parapsilosis	8.08±0.08^{c}	7.54±0.27^{c}	6.57±0.22^{c}	6.53±0.39^{c}
P. flavipulchra	1.60±0.2^{a}	1.13±0.23^{a}	0.42±0.23^{a}	0.23±0.04^{a}
L. sakei	0.94±0.16^{a}	0.73±0.09^{a}	0.48±0.08^{a}	0.38±0.01^{a}
B. natto	6.05±0.25^{b}	4.41±0.5^{b}	2.33±0.44^{b}	1.71±0.14^{b}

Rotifers *Brachionus plicatilis* fed on different strains were evaluated separately in order to calculate mean population growth (Table 2). The results demonstrated that there were significant differences among the treatments (p<0.05). Rotifers that fed on *L. sakei* (120.9±6.22 individual/mL) and *B. natto* (122.14±6.68 individual/mL) showed maximum population growth. The results of daily counting of the population are shown in figure 1, which demonstrate greater population growth in the treatments that fed on *L. sakei* and *B. natto.*

In Table 3, the results related to the enzymes activity assay are presented. These results showed a significant increase in all experimental treatments in comparison with the control (p<0.05). The highest rate of amylase (1.37±0.12 U/mg protein min), lipase (0.92±0.02 U/mg protein min) and protease (1.76±0.42 U/mg protein min) were recorded in the treatment that fed on *B. natto*. In lipase activity, no significant difference was observed between *B. natto* and *P. flavipulchra* (p>0.05). Also in protease activity, *B. natto* was close to *L. sakei* and *C. parapsilosis*, and no significant difference was observed between these three treatments. The lowest lipase activity was recorded in the treatment that fed on *C. parapsilosis*, which was even lower than the control (p<0.05).

Table 2: Mean value (±SE) of population indices of *Brachionus plicatilis* which were fed on different bacteria and yeasts. Different letters in the same column indicate significant differences (p<0.05). PIR refers to population increase rate (per day); GIR refers to generation increase rate (per day).

	Mean population	**PIR**	**GIR**
Control	87±2.78^{b}	6.9±0.99^{d}	4.7±0.48^{c}
C. parapsilosis	124.14±3.54^{a}	12.76±1.45^{c}	7.13±0.5^{b}
P. flavipulchra	52.61±2.69^{c}	-3.52±0.26^{e}	-4.79±0.49^{d}
L. sakei	120.9±3.59^{a}	34.19±1.23^{a}	12.28±0.21^{a}
B. natto	122.14±3.86^{a}	26.61±2.92^{b}	10.81±0.61^{a}

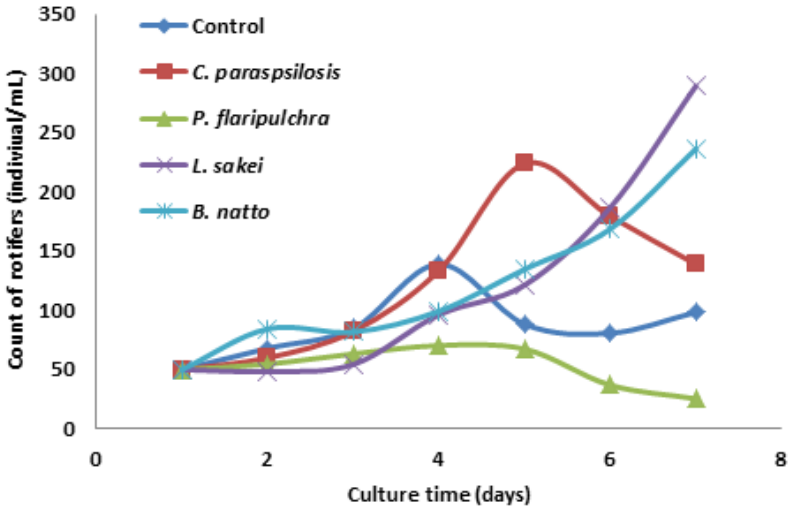

Figure 1: Population changes of rotifers fed on different bacteria and yeast over seven days. Data expressed as mean ± SD (n=12).

Table 3:Enzymes activity of *Brachionus plicatilis* fed on different microbial strains. Different letters in the same column indicate significant differences (p<0.05).

	Enzymes activity (U/mg protein min)		
	α amylase	**Lipase**	**Protease**
Control	0.42±0.03^{b}	0.54±0.09^{b}	0.46±0.03^{d}
C. parapsilosis	0.59±0.01^{b}	0.34±0.03^{c}	0.81±0.02^{b}
P. flavipulchra	0.66±0.02^{b}	0.87±0.04^{a}	0.60±0.02^{c}
L. sakei	0.69±0.02^{b}	0.59^{b}	1.09±0.10^{ab}
B. natto	1.37±0.12^{a}	0.92±0.02^{a}	1.76±0.42^{a}

The nutritional quality of *Brachionus plicatilis* biomass produced under laboratory condition showed significant differences in biochemical characteristics. The results presented in Table 4 show significant differences between treatments and the control. The highest crude protein belonged to all four treatments that fed on different beneficial microorganisms (p<0.05). The highest lipid was observed in treatment that fed on *B. natto* (11.89±0.05%), and the lowest belonged to *C. parapsilosis* (9.93±0.02%) and *L. sakei* (9.61±0.13%). Bacterium *P. flavipulchra* caused the highest carbohydrate and ash percentage (p<0.05). The highest percentage of moisture was recorded in the treatment fed on *B. natto* and control treatment.

Microbial longevity was evaluated to find out the count of applied strains after 2, 4, and 8 days of enrichment. The results, shown in figure 2, demonstrated that except *B. natto* the rest of the strains decreased longevity with suspension in inoculation.

Table 4: Chemical composition of *Brachionus plicatilis* enriched with different microorganisms. Different letters in the same column indicate significant differences ($p<0.05$).

	Body composition (%)				
	Protein	**Lipid**	**Carbohydrate**	**Ash**	**Moisture**
Control	44.67 ± 0.22[b]	10.76 ± 0.14[b]	13.66 ± 0.13[d]	20.59 ±0.06[a]	87.8 ± 0.15[a]
C. parapsilosis	48.56 ± 0.28[a]	9.93 ± 0.02[c]	17.56 ± 0.17[b]	18.79 ± 0.09[b]	70[c]
P. flavipulchra	49.03 ± 0.51[a]	10.79[b]	19.35 ± 0.23[a]	19.9 ±0.23[ab]	70.1 ± 0.23[c]
L. sakei	50.7 ± 0.12[a]	9.61 ±0.13[c]	15.16 ± 0.31[c]	20.38 ± 0.23[a]	86[a]
B. natto	49.83 ± 0.03[a]	11.89 ±0.05[a]	14.68 ±0.1[cd]	18.64 ± 0.2[b]	81.56 ± 0.38[b]

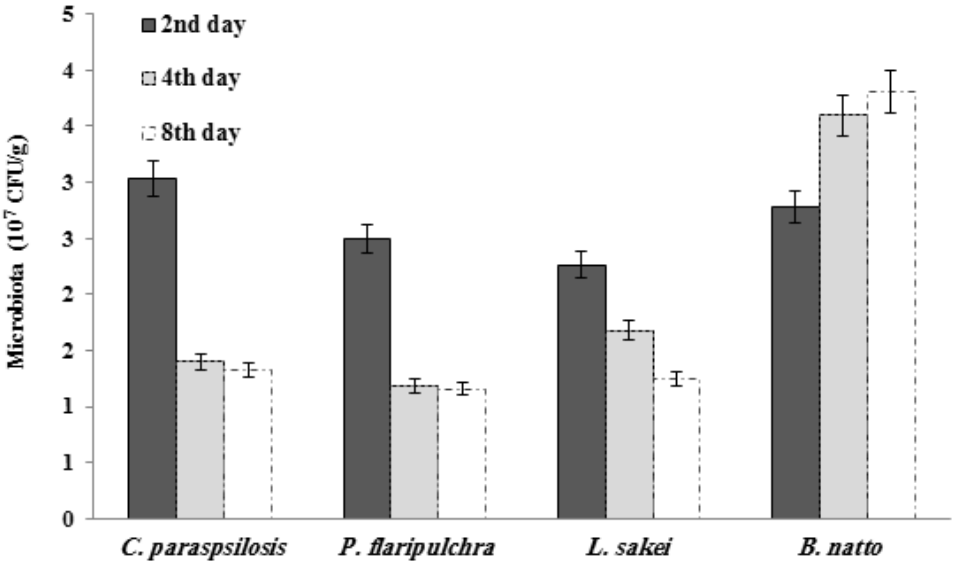

Figure 2: Different bacterial and yeast strains longevity in *Brachionus plicatilis* over 8 days. Values expressed in mean ± SD.

Microbial labeling with blue fluorescent stain showed a high latent inoculation of applied probiotics in rotifers over three hours of enrichment (Fig. 3). The results demonstrated that beside microbial ingestion the adhesion of applied bacteria and yeast was observed as well, which adhered to the lorica of rotifers. Micrographs also confirmed microbial ingestion by rotifers in a way that labeled strains accumulated in the jaws, stomach, and gastric glands.

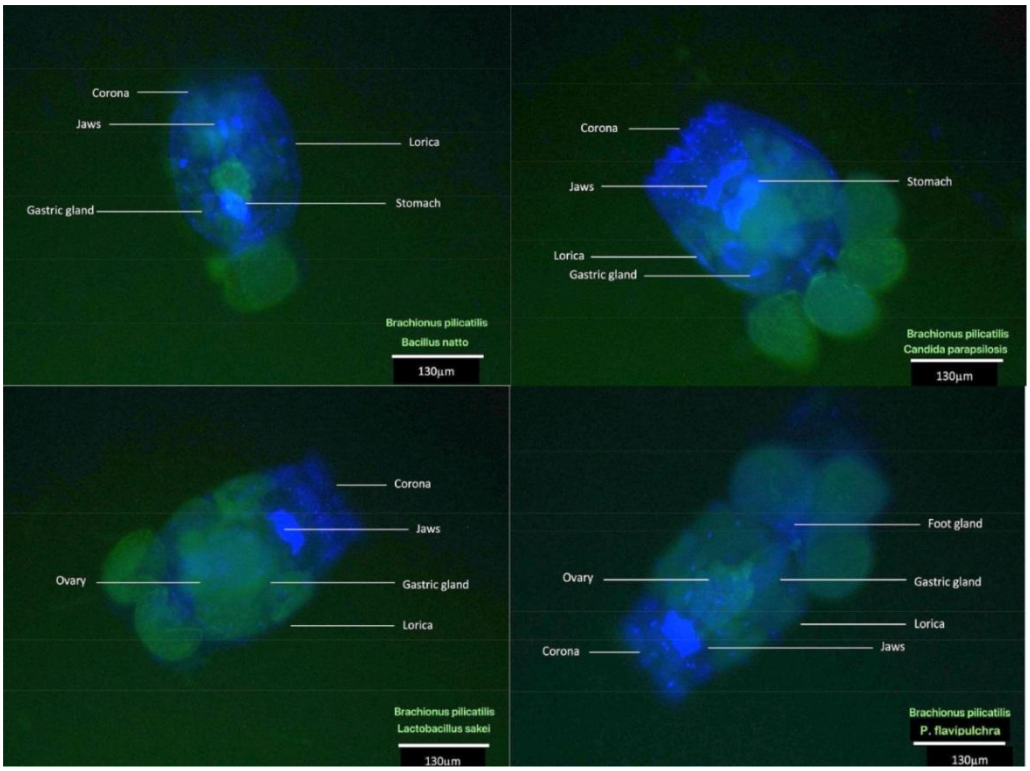

Figure 3: Different bacterial and yeast strains inoculation forms in *Brachionus plicatilis* over 3 hours of enrichment. The blue colour parts refer to the accumulation of labeled microbes in rotifers.

Discussion

The results of the present study showed that all the applied strains significantly suppressed the count of *Vibrio spp*. Susceptibility test of the used strains was the primary step for finding the beneficial strains to suppress the *Vibrio* that was carried out and reported before (Sahandi *et al.*, 2019). Application of selected strains in enrichment of rotifers was the next purpose that followed in this study. This study focused on finding the best strain which besides suppression of *Vibrio*, improves population growth as well as retention of nutrients in the rotifers body. The three strains, including *P. flavipulchra*, *B. natto*, and *L. sakei*, significantly reduced the count of *Vibrio* species over 20 hours after inoculation time. Between these three strains, the lowest count of *Vibrio* was observed in the treatment enriched with *P. flavipulchra* ($0.23\pm0.04\times10^7$CFU/g). Other two strains including, *L. sakei* ($0.38\pm0.01\times10^7$CFU/g) and *B. natto* ($1.71\pm0.14\times10^7$CFU/g), significantly reduced the count of *Vibrio* which was ingested by rotifers when compared with *C. parapsilosis* ($6.53\pm0.39\times10^7$CFU/g) and the control ($14.09\pm0.32\times10^7$CFU/g). The bacterial and yeast strains which were used in this study, successfully inhibited four pathogenic *Vibrio* including, *Vibrio anguillarum*, *V. campbellii*, *V. harveyi*, *V. parahaemolyticus*, in a susceptibility test (Sahandi *et al.*, 2019). The fluorescent labeling and microscopy of these bacteria and yeast showed that the rotifers successfully ingested all the four strains which were accumulated in jaws, stomach, and gastric glands (Figure 3). Yu *et al.* (2012) reported that *P. flavipulchra* G1 has

latent secreting antibacterial compounds which could inhibit *Vibrio spp*. There are other reports such as Chen *et al.* (2016) and Georgieva *et al.* (2015), which showed antibiotic activity of *Bacillus* and *Lactobacillus* against different *Vibrio* species. For this investigation, benzalkonium chloride solution was used to disinfect the outer side of rotifers, so the achieved results were related to the ingested *Vibrio* species by rotifers (p<0.05). The great inoculation of probiotics in live food refers to well ingestion and adhesion. These two conditions are supplements of each other to cause a proper result. Maximum interaction between pathogenic and probiotic strains is happening inside of the digestive tract. However, fluorescent microscopy showed that applied strains, beside sufficient ingestion, could adhere to the lorica, eggs, and tail of rotifers. Moriarty (1998) reported that bacteria could produce antibiotic compounds to compete for nutrients and sites. Sufficient suppression of *Vibrio* by *L. sakei*, *P. flavipulchra* and *B. natto* could be because of successful adhesion of these bacteria in epithelial cells of the digestive tract and successful forming of biofilm which suppress the loading of *Vibrio*. How the applied bacteria suppress *Vibrio* needs to be considered. The suppression caused by *P. flavipulchra* might be because of antimicrobial and antagonist components secreted by this strain. *Bacillus* species secreting extracellular enzymes (Jafaryan *et al.*, 2011; Sahandi *et al.*, 2012) and *Lactobacillus* by secretion of organic acids (Farzanfar, 2006) might suppress *Vibrio* species.

Rotifers are among the most widely used aquaculture live food because they are convenient to use and are readily available (Dhert *et al.*, 2001; Sahandi and Jafaryan, 2011). Culture of rotifers is entirely different from that of *Artemia*. *Artemia* may be produced by use of cysts, which is so easy to reach to high concentrations, just in 24 hours. However, in rotifers, this is a little bit different. For the culture of rotifers, usually, the first stock is introduced into culture tanks or ponds and the rotifers are harvested by plankton net or by leading them as whole of the culture to the host organism tank. The primary source of pathogenic bacteria in mariculture is water supply. So inoculation of pathogenic bacteria such as *Vibrio* is unpreventable, however with use of beneficial bacteria it is possible to reduce pathogens and increase the nutritional quality of rotifers which is suggested by several researchers (Planas *et al.*, 2004; Sahandi and Jafaryan, 2011; Sahandi *et al.*, 2012). Using beneficial bacteria in suppression of *Vibrio* does not necessarily increase yield, in contrast, may reduce the yield as well. This is because of the different effects that a bacterium may have on the host organism. So one must consider all aspects of probiotics effects on the host organism, not just a single purpose.

In the present study, the use of different strains caused significant growth in the population of rotifers when compared with the control over 7 days (p<0.05). The results demonstrated that population of rotifers that fed on *C. parapsilosis* (124.14±6.14 rotifer/mL; r=12.79±2.51; $g.t$=7.13±0.86) were significantly bigger

than others, but as it is presented in Table 1 the use of this yeast strain caused the lowest suppression. This is clear that yeast contains nutrients that may provide proper conditions for microbial growth and consequently increase the count of bacteria in host organisms, whether pathogenic or probiotic (Jafaryan *et al.*, 2011; Jafaryan *et al.*, 2014). Besides, the fluorescent microscopy showed the presence of *C. parapsilosis* in stomach and jaws of rotifers, which approved the high ability of rotifers in taking the yeast cells (Fig. 3). Also using *C. parapsilosis* after day 5 reduced the population of rotifers like what was reported by Sahandi and Jafaryan (2011) (Fig. 1). Tamaru *et al.* (1993) suggested using a combination of yeast and microalgae for increasing the growth rate of rotifers, and this was reported again by Sahandi and Jafaryan (2011). However, in the mentioned studies, count of pathogens in rotifers was not considered. Using *B. natto* (122.14±6.68 rotifer/mL; r=26.61±5.07; $g.t$=10.81±1.05) significantly increased the rotifers population growth (p<0.05, Table 2). The graph of population growth which is presented in Figure 1 also showed the increase of population in rotifers that were cultured by addition of *B. natto.* In contrast the use of *P. flavipulchra* (52.61±4.66 rotifer/mL; r=-3.52±.45; $g.t$=-4.79±0.86) reduced the growth of rotifers (Table 2; Fig. 1). Yu *et al.* (2012) reported that *P. flavipulchra* JG1 could synthesize various antimicrobial metabolites, including protein and small molecules. These antimicrobial components may affect rotifers and reduce their population.

The enzymes activity in rotifers were increased in the treatment that fed on *B. natto* (p<0.05). Beneficial bacteria and yeast may cause this increase in amylase, lipase, and protease activity. Similar results were reported by De Schrijver and Ollevier (2000). Arellano-Carbajal and Olmos-Soto, (2002) reported that probiotics effectively participate in digestion due to the production of extracellular enzymes such as protease, lipase, and carbohydrases, which would end to the high growth rate in the host organism. The lowest enzymes activity was observed in control (p<0.05). However, the greatest performance of the enzyme's activity was observed in the treatment fed on *B. natto* (Table 3). Extracellular enzymes secreted by *Bacillus* species might increase the enzymes activity in rotifers.

The use of different bacteria and yeast caused significant differences in enzymes activity of rotifers when compared with the control (p<0.05). The increase in enzymes activity would result in increased digestion, which would result in the utilization of more nutrients. In our study, the highest crude protein was observed in two treatments, *L. sakei* (50.7±0.1%), and *B. natto* (49.83±0.03%). But the highest crude fat was just recorded in *B. natto* (11.89±0.05%). This could be because of the secretion of different types of extracellular enzymes by *Bacillus* species. As it was mentioned before lipase activity (0.92±0.02 U/mg protein/min) in rotifers that fed on *B. natto* was higher than those of the other groups. Farzanfar (2006) reported that probiotics could break down

protein and carbohydrate; therefore; they can cause an increase of nutrient retention and increase the quality of body composition of the host organism. Wang and Xu (2006) suggested that addition of probiotics improve digestibility, which might, in turn, explain better growth performances. It is reported that bacteria with secreting protease can break down peptide bonds and form free amino acids (MacFarlane and Cummings, 1991). These amino acids are absorbed quickly and retained in the host organism's body as protein. Probiotics can break down protein and carbohydrate (Sarem-Damerdjii *et al.*, 1995) therefore; they can increase nutrient retention and carcass chemical composition. Probiotics enhance the population of useful bacteria and influence the digestive process by improving intestinal microbiota, consequently improving digestibility and absorption of nutrients (Ghosh *et al.*, 2002; Jafaryan *et al.*, 2011).

Long-term inoculation of selected strains in rotifers was investigated over 8 days. Successful inoculation after addition of beneficial bacteria and longevity of them in the digestive system is an important property which would ensure the success of probiotics in the digestive tract for a long time. The *B. natto* count started to increase after 8 days of starvation (p<0.05; Fig. 2). Successful long-term inoculation of *Bacillus* strains may happen due to their reproduction form. *Bacillus* strains multiply by production of spore. Therefore, probiotics enhanced the population of beneficial bacteria and influenced the digestive process by improving intestinal microbiota, consequently improving digestibility and absorption of nutrients (Bomba *et al.*, 2002). Long-term inoculation failure of other strains might have different reasons. Ghomi *et al.* (2010) reported that for successful inoculation of *Lactobacillus* species, they must be used permanently. This reason might be considered for *C. parapsilosis* and *P. flavipulchra* as well.

Increasing probiotics usage in aquaculture is an important issue worldwide. This is because of the different range of effects that may be caused by various bacteria or yeast species. Finding a single strain that could affect different areas such as reducing pathogens and increasing growth and yield is vital for the future of aquaculture. It is crucial to determine the aim (single or multiple) before any plan for the use of beneficial bacteria or yeast as probiotic. The results of this study confirmed the positive effect of *B. natto* and *L. sakei* on suppression of *Vibrio*, increased population growth, increased enzymes activity, and body composition in rotifers. However, the strain of *B. natto* was the only strain that showed long-term inoculation which must be considered. Successful inoculation of *B. natto* of sure would have economic benefits for farmers. Although still further studies are required for explaining the mechanism of these two strains' effects.

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